J. Mol. Biol. (1996) **263**, 432–446

JMB

Assembly of the Herpes Simplex Virus Capsid: Characterization of Intermediates Observed During Cell-free Capsid Formation

William W. Newcomb[1], Fred L. Homa[2], Darrell R. Thomsen[2] Frank P. Booy[3], Benes L. Trus[4], Alasdair C. Steven[3] Juliet V. Spencer[1] and Jay C. Brown[1]*

[1]*Department of Microbiology and Cancer Center University of Virgina Health Sciences Center Charlottesville, VA 22908 USA*

[2]*Molecular Biology Research Pharmacia & Upjohn Inc. Kalamazoo, MI 49001, USA*

[3]*Laboratory of Structural Biology, National Institute of Arthritis, Musculoskeletal and Skin Diseases and* [4]*Computational Bioscience and Engineering Laboratory Division of Computer Research and Technology National Institutes of Health Bethesda, MD 20892-2755 USA*

**Corresponding author*

The herpes simplex virus-1 (HSV-1) capsid is an icosahedral shell approximately 15 nm thick and 125 nm in diameter. Three of its primary structural components are a major capsid protein (VP5; coded by the UL19 gene) and two minor proteins, VP19C (UL38 gene) and VP23 (UL18 gene). Assembly of the capsid involves the participation of two additional proteins, the scaffolding protein (UL26.5 gene) and the maturational protease (UL26 gene). With the goal of identifying morphological intermediates in the assembly process, we have examined capsid formation in a cell-free system containing the five HSV-1 proteins mentioned above. Capsids and capsid-related structures formed during progressively longer periods of incubation were examined by electron microscopy of thin-sectioned specimens. After one minute, 90 minutes and eight hours of incubation the structures observed, respectively, were partial capsids, closed spherical capsids and polyhedral capsids. Partial capsids were two-layered structures consisting of a segment of external shell partially surrounding a region of scaffold. They appeared as wedges or angular segments of closed spherical capsids, the angle ranging from less than 30° to greater than 270°. Partial capsids are suggested to be precursors of closed spherical capsids because, whereas partial capsids were the predominant assembly product observed after one minute of incubation, they were rare in reactions incubated for 45 minutes or longer. Closed spherical capsids were highly uniform in morphology, consisting of a closed external shell surrounding a thick scaffold similar in morphology to the same layers seen in partial capsids. In negatively stained specimens, closed spherical capsids appeared round in profile, suggesting that they are spherical rather than polyhedral in shape. A three-dimensional reconstruction computed from cryoelectron micrographs confirmed that closed spherical capsids are spherical with $T = 16$ icosahedral symmetry. The reconstruction showed further that, compared to mature HSV-1 capsids, closed spherical capsids are more open structures in which the capsid floor layer is less pronounced. In contrast to closed spherical capsids, polyhedral capsids exhibited distinct facets and vertices, indicating that they are icosahedral like the capsids in mature virions. Upon incubation *in vitro*, purified closed spherical capsids matured into polyhedral capsids, indicating that the latter arise by angularization of the former. Partial capsids, closed spherical capsids and polyhedral capsids were all found to contain VP5, VP19C, VP23, VP21 and the scaffolding protein; the scaffolding protein being predominantly in the immature, uncleaved form in all cases. Polyhedral capsids and closed spherical capsids were found to differ in their sensitivity to disruption at 2°C. Closed spherical capsids were disassembled while polyhedral capsids were unaffected. Our results suggest that HSV-1 capsid assembly begins

Abbreviations used: HSV, herpes simplex virus; PBS, phosphate-buffer saline; dsDNA, double-stranded DNA.

0022–2836/96/430432–16 $25.00/0

with the partial capsid and proceeds through a closed, spherical, unstable capsid intermediate to a closed, stable, icosahedral form similar to that found in the mature virion. Structures resembling HSV-1 partial capsids have been described as capsid assembly intermediates in *Salmonella typhimurium* bacteriophage P22. HSV-1 capsid maturation from a fragile, spherical state to a robust polyhedral form resembles the prohead maturation events undergone by dsDNA bacteriophages including λ, T4 and P22. Because of this similarity, we propose the name procapsid for the closed spherical capsid intermediate in HSV-1 capsid assembly.

Keywords: herpes simplex virus; capsid assembly; electron microscopy; scaffolding protein; procapsid

Introduction

Like other members of the herpesvirus family, herpes simplex virus-1 (HSV-1) consists of an icosahedral capsid surrounded by a membrane envelope. The virus DNA is contained inside the capsid, and a layer of protein, called the tegument, is found between the capsid and the membrane creating a virion with an overall diameter of approximately 200 nm (Dargan, 1986; Roizman, 1990).

The structure of the HSV-1 capsid has been determined to resolutions in the range of 1.9 nm to 3.0 nm by cryoelectron microscopy and three-dimensional image reconstruction (Schrag *et al*., 1989; Booy *et al*., 1991; Newcomb *et al*., 1993; Zhou *et al*., 1994, 1995). Such reconstructions show that the capsid is a protein shell approximately 15 nm thick and 125 nm in diameter. Its major structural features are 162 capsomers (150 hexons and 12 pentons) that lie on a $T = 16$ icosahedral lattice. The pentons have 5-fold rotational symmetry and are located at the capsid vertices while the hexons have 6-fold symmetry and are found on the edges and faces of the capsid icosahedron. The capsomers are connected in groups of three by structures called triplexes (320 in all) that lie on the capsid floor at the base of the capsomer protrusions. One triplex is found at each local 3-fold position where it makes contact with each of the three adjacent capsomers.

Compositionally, the most prominent components of the capsid are the major capsid protein (VP5; M_r 149,075; product of the UL19 gene) and three minor proteins, VP19C (M_r 50,260; UL38 gene), VP23 (M_r 34,268; UL18 gene) and VP26 (M_r 12,095; UL35 gene) (Gibson & Roizman, 1972; Newcomb *et al*., 1993; Rixon, 1993). VP5 is the structural subunit of the capsomers, both the hexons and the pentons; pentons each contain five copies of VP5 while hexons have six (Newcomb *et al*., 1993). VP19C and VP23 make up the triplexes. On average, triplexes contain one copy of VP19C and two of VP23, although triplex composition may vary with position in the capsid shell (Newcomb *et al*., 1993; Zhou *et al*., 1994). VP26 is located at the distal tips of the hexons with one VP26 molecule bound to each hexon-associated VP5 (Booy *et al*., 1994; Trus *et al*., 1995; Zhou *et al*., 1995). Table 1 summarizes information about the gene and protein names for the most abundant HSV-1 capsid proteins. HSV-1 capsids contain smaller amounts of other proteins including the maturational protease (VP24; a product of gene UL26) and the product of gene UL6 (Rixon, 1993; Newcomb *et al*., 1993; Patel & MacLean, 1995).

HSV-1 capsids are formed in the infected cell nucleus. DNA-free, closed capsid shells first self-assemble in the nucleus and are later packaged with DNA, following a pathway similar to that of dsDNA bacteriophages (Casjens & Hendrix, 1988; Roizman & Sears, 1990; Rixon, 1993). In addition to the structural proteins mentioned above, assembly of the HSV-1 capsid involves the participation of the scaffolding protein (the product of the UL26.5 gene), and the maturational protease (UL26). During capsid assembly, the scaffolding protein forms a core (the scaffold) internal to the capsid shell and interacts directly with VP5 in the shell (Thomsen *et al*., 1995; Hong *et al*., 1996). The scaffolding protein participates in capsid assembly but it is not present in the mature HSV-1 virion. Activity of the maturational protease is required for assembly of DNA-containing capsids (Preston *et al*., 1983; Gao *et al*., 1994). The full-length (635 amino acids) protease cleaves itself in two places, an R site (Ala247-Ser248) to separate VP24 (Met1-Ala247) and VP21 (Ser248-Arg635), and an M site (Ala610-Ser611) whose products are the cleaved form of VP21 (Ser248-Ala610) and a 25 amino acid peptide (Ser611-Arg635) (DiIanni *et al*., 1993; Liu & Roizman, 1993; Weinheimer *et al*., 1993). Since the UL26.5 gene overlaps (in frame) the C-terminal 329

Table 1. HSV-1 genes encoding proteins involved in capsid assembly

Gene	Protein poduct(s)	Comment
UL18	VP23	Component of triplexes
UL19	VP5	Major component of capsomers
UL26	VP21, VP24	Protease activity (VP24)
UL26.5	preVP22a	Scaffolding protein
UL35	VP26	Hexon tips
UL38	VP19C	Component of triplexes

residues coded by UL26, the M site is also found in the scaffolding protein where it is cut by the protease releasing VP22a (Met1-Ala304; also called ICP35e,f) and the C-terminal 25 amino acid peptide (Ser305-Arg329) (Preston *et al*., 1992; Liu & Roizman, 1995).

Recent studies of HSV-1 capsid assembly have made use of a panel of six recombinant baculoviruses each of which codes for one of six proteins (VP5, VP19C, VP23, VP26, the scaffolding protein and the maturational protease) involved in capsid formation. The panel of recombinant baculoviruses has provided the basis for methods to study HSV-1 capsid formation in Sf9 cells multiply infected with recombinant baculoviruses (Tatman *et al*., 1994; Thomsen *et al*., 1994) and in cell-free extracts (Newcomb *et al*., 1994). Studies with the two systems have defined the minimum set of HSV-1 genes (UL18, UL19, UL38, and either UL26 or UL26.5) required for formation of closed, icosahedral capsids. Both systems are well-suited for further analysis of capsid assembly and we have been pursuing studies with both (Thomsen *et al*., 1995). Here we report the results of experiments in which the cell-free system was examined for the presence of morphological intermediates in the assembly process. Capsids and capsid-related structures formed in the cell-free system were harvested at different times after the initiation of assembly and analyzed by electron microscopy and SDS-polyacrylamide gel electrophoresis. The results have suggested the nature of two structures, partial capsids and closed spherical capsids, that appear to be involved as intermediates in formation of the mature capsid icosahedron.

Results

To identify structural intermediates in the assembly of HSV-1 capsids, we employed the cell-free capsid assembly system recently described by Newcomb *et al*. (1994). Separate samples of recombinant baculovirus-infected Sf9 cells containing the proteins coded by HSV-1 genes UL18, UL19, UL26, UL26.5 and UL38, respectively, were mixed, lysed by freezing and thawing, adjusted to 5 mM DTT, 50 mM EDTA and incubated. Capsids and capsid-related structures formed during the incubation period were precipitated from reaction mixtures by addition of a monoclonal antibody (6F10) specific for VP5. Precipitates were then analyzed by electron microscopy and SDS-polyacrylamide gel electrophoresis.

Partial capsids

Except for a small proportion of closed capsids, partial capsids were the only recognizable assembly products observed in reaction mixtures incubated at 28°C for one minute (plus an additional 15 minutes at room temperature for antibody precipitation). In electron micrographs of thin-sectioned specimens (Figure 1a and b), partial capsids were seen to consist of a darkly staining shell layer partially surrounding a region of more lightly staining material (the scaffold). Partial capsids appeared as wedges or angular segments of closed capsids, the extent of the angular segment ranging from approximately 30° to greater than 270°. The shell and scaffold regions of partial capsids bore a close morphological resemblance to the respective regions of closed spherical capsids (compare Figure 1a and c). The thickness of the shell layer and its curvature, for example, were the same in partial capsids and closed spherical capsids. In most images of partial capsids, the ratio of shell to scaffold material was approximately the same as the ratio found in closed spherical capsids. It was rare to see a nearly complete capsid shell with only a small amount of scaffold or a large amount of scaffold with a small amount of shell. In some images, short projections from the scaffold could be seen to make contact with the shell layer (see Figure 1b, arrow).

In addition to partial capsids, thin-sectioned preparations of one minute reaction products showed amorphous material in which partial capsids appeared to be embedded (Figure 1a). This material was not characterized further. We assume it consists of capsid proteins that have not yet become organized into recognizable structures.

The organization of partial capsids into distinct shell and scaffold layers was also observed, in negatively stained specimens (Figure 2a). In such preparations the shell could occasionally be seen to consist of capsomer-sized subunits (Figure 2a, arrow) which appeared to make contact with the scaffold.

Closed spherical capsids

Partial capsids and closed spherical capsids were the only assembly products observed in reaction mixtures incubated between one and 90 minutes, with the proportion of closed spherical capsids increasing as a function of incubation time. By 45 minutes of incubation, closed spherical capsids accounted for greater than 90% of the capsid-related structures present and this high proportion persisted for up to 90 minutes of incubation (Figure 1c). Closed spherical capsids were highly uniform in structure with distinct shell and scaffold layers (Figure 1c). The morphology of the shell was similar to that seen in partial capsids, but the scaffold appeared more ordered. In most cases it was seen as a distinct inner shell rather than the less structured material found in partial capsids. Closed spherical capsids were quite uniform in size with the diameter measured from thin sections (103.6 $\pm$ 4.0 nm [n = 38]) similar to that of HSV-1 B capsids (104.5 $\pm$ 4.0 nm [n = 24]).

Visualization of negatively stained specimens confirmed that closed spherical capsids were highly uniform in morphology (Figure 2b). Closed spherical capsids appeared circular in profile, suggesting that they are spherical in shape rather

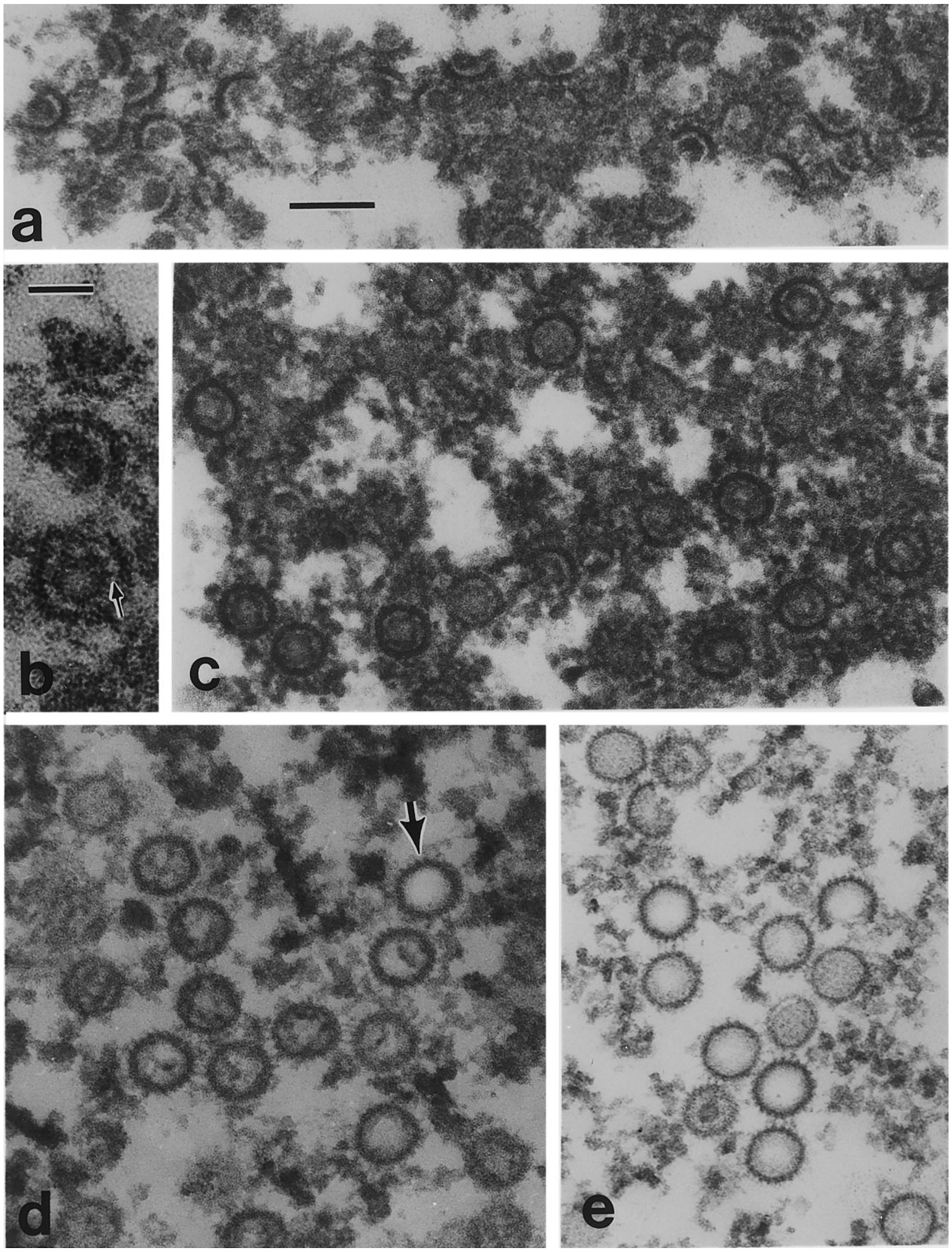

Figure 1. Electron microscopy of capsid assembly products recovered from cell-free reaction mixtures and thin-sectioned. Assembly products were isolated by antibody precipitation from reaction mixtures incubated as follows: a and b, one minute at 28°C; c, 90 minutes at 28°C; d, eight hours at 28°C; e, eight hours at 37°C. Thin-sectioned preparations were prepared for electron microscopy as described in Materials and Methods. Note that the structures shown in a and b, called partial capsids, consist of distinct scaffold and shell layers. The arrow in b indicates a connection (several others are visible) between the scaffold and shell. In d the arrow indicates a capsid that contains a shell but no scaffold. Note that most capsids in e lack a scaffold. All panels except b are printed at the same magnification. Bar represents (a) 150 nm; (b) 70 nm.

than polyhedral as observed in more mature HSV-1 capsids (compare Figure 2b with c or d). No evidence of facets or vertices was seen in capsids isolated from 90 minute incubations. Capsomers were seen to be present on the surface of closed spherical capsids and they appeared to be similar in number and morphology to those observed in comparable images of icosahedral HSV-1 capsids.

The structure of closed spherical capsids was examined further by cryoelectron microscopy and three-dimensional image reconstruction. In cryoelectron micrographs (Figure 3a), closed spherical capsids appeared highly uniform in morphology as they did in negatively stained preparations. They were round in profile with distinct scaffold and shell layers. In most images the scaffold appeared as a ring whose diameter was constant from capsid to capsid. Some capsids lacking scaffolds were observed (Figure 3a, arrow), but they were rare.

A three-dimensional reconstruction of the closed spherical capsid was computed from cryoelectron micrographs (such as that shown in Figure 3a) at a

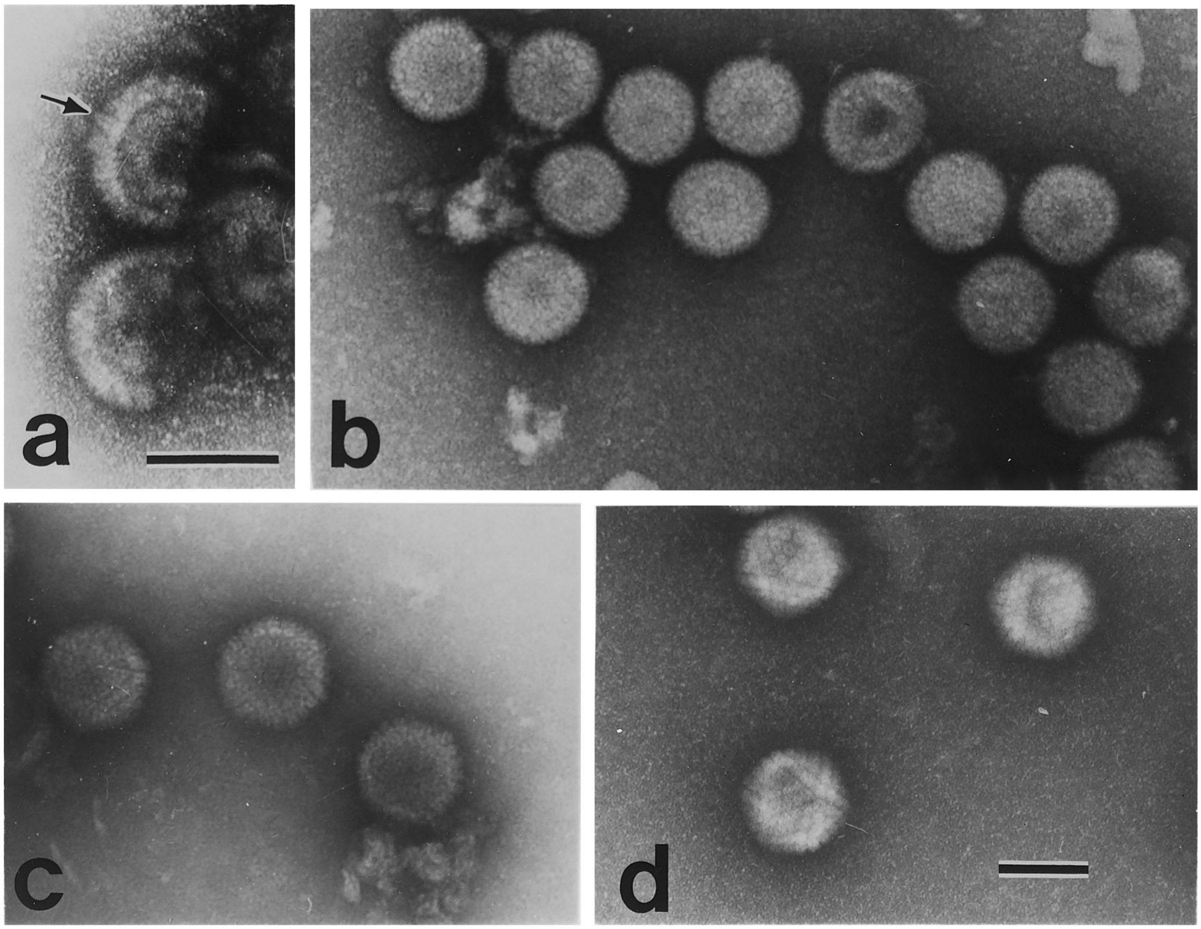

Figure 2. Electron microscopy of assembly products recovered from cell-free reaction mixtures and negatively stained with uranyl acetate. Assembly products were isolated by antibody precipitation from reaction mixtures incubated under the following conditions: a, one minute at 28°C; b, 90 minutes at 28°C; c, eight hours at 28°C; d, 90 minutes at 28°C followed by four hours at 37°C as described in the text. Negative staining with uranyl acetate and electron microscopy were performed as described in Materials and Methods. Note that facets and vertices are present in the capsids shown in c and d. b–d are printed at the same magnification. Bar represents 100 nm.

resolution of 2.7 nm as described in the accompanying paper (Trus *et al*., 1996). A total of 88 capsid images was used in the computation. The reconstruction, shown in a surface view in Figure 3c, confirmed that the closed spherical capsid is spherical in morphology with a diameter of 125 nm. It can be seen to consist of hexons and pentons lying on a $T = 16$ icosahedral lattice as in the mature HSV-1 capsid. The round morphology of the closed spherical capsid is the most conspicuous structural difference between it and the mature, polyhedral HSV-1 capsid, but there are other important differences. For example, around and between the capsomers there are additional holes not found in the mature capsid, making the closed spherical capsid an open, quite porous structure. The capsomer subunits are more elongated in the closed spherical capsid, with pointed rather than flat surfaces at their distal ends. Further, in the closed spherical capsid the capsomer subunits are less closely associated with each other than they are in the mature capsid. Material is present at the local 3-fold positions (Figure 3c), the sites occupied by the triplexes in the mature capsid. Further information about the closed spherical capsid structure is presented in the accompanying paper (Trus *et al*., 1996).

Polyhedral capsids

The assembly products observed after eight hours of incubation were closed capsid shells that were highly uniform in diameter (Figure 1d). The shell layer was similar in morphology to that seen in closed spherical capsids, and electron micrographs showed that most eight hour capsids had some scaffold material. Compared to the scaffold material seen in closed spherical capsids, however, the scaffold in eight hour capsids appeared less organized and lower in total amount (compare Figure 1d with c). Some eight hour capsids lacked scaffold material entirely (arrow in Figure 1d). In one representative experiment, for example, 23 of 200 (11.5%) eight hour capsids had no scaffold

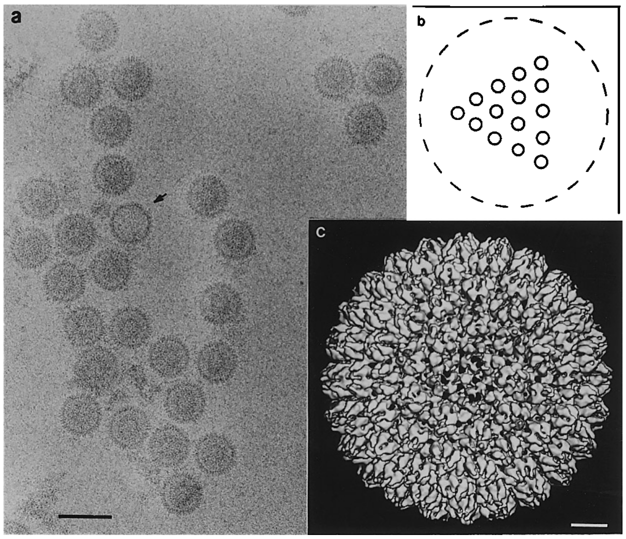

Figure 3. Cryoelectron microscopy and three-dimensional image reconstruction of the closed spherical capsid. Specimens were prepared for cryoelectron microscopy by precipitation from reaction mixtures with antibody 6F10 as described in Materials and Methods. The accompanying paper (Trus *et al*., 1996) describes the procedures employed for cryoelectron microscopy and image reconstruction. Cryoeoectron micrographs (a) show that closed spherical capsids are uniform in diameter with distinct shell and scaffold layers, the scaffold appearing as a ring in most images. Capsids lacking scaffold material (arrow) were rare. A surface-shaded representation of the three-dimensional reconstruction, viewed along the 3-fold axis of rotational symmetry, is shown in c. Features of the capsid structure can be identified with reference to the diagrammatic representation in b. The broken curve shows the profile of the capsid while the small circles indicate the positions of capsomers (pentons at the vertices of the triangle with hexons elsewhere). Note that capsomers consist of column-shaped subunits with points at the distal ends and thickenings at the middle. Triplexes can be seen at local 3-fold positions among groups of three capsomers. For example, the viewer is looking straight down at the triplex on the icosahedral 3-fold position. Bar represents (a) 150 nm; (c) 15 nm.

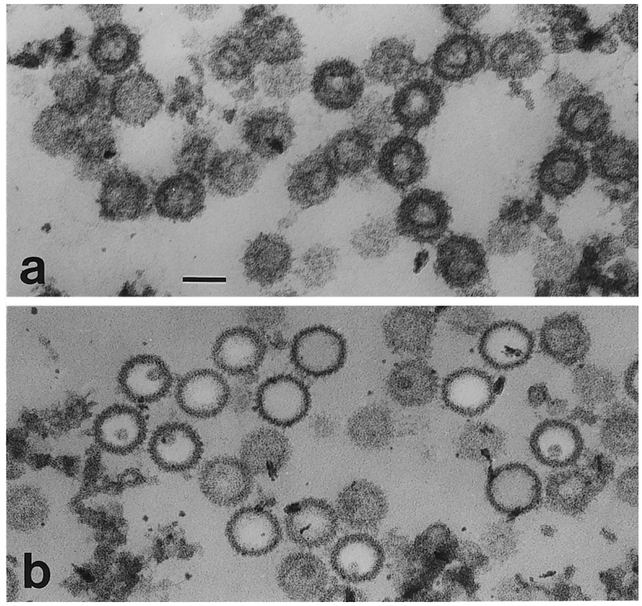

Figure 4. Electron microscopy of closed spherical capsids (a) and the polyhedral capsids that result when closed spherical capsids are incubated at 37°C for four hours (b). Closed spherical capsids were obtained from reaction mixtures that were constituted and incubated for 90 minutes at 28°C as described in Materials and Methods. Polyhedral capsids were formed beginning with a sample of the closed spherical capsid preparation shown in a. This preparation was incubated in PBS containing 20 mM $MgCl_2$ for four hours at 37°C as described in the text. Note that after incubation at 37°C capsids are polyhedral rather than spherical in shape and that they have a shell layer, but no scaffold or a very small scaffold. Bar represents 80 nm.

material. In negatively stained preparations, eight hour capsids yielded images suggesting that they are polyhedral rather than spherical in shape. They appeared angular instead of circular in profile (Figure 2c; compare Figure 2b) and distinct facets and vertices were often observed. Facets and vertices can be seen, for example, in the leftmost two capsids in Figure 2c. Capsomers could be distinguished in many images, and they were found to be hexons and pentons as in native HSV-1 capsids.

Assembly products were examined in reaction mixtures incubated for up to 18 hours at 28°C. In thin sectioned preparations, the capsids observed in eight hour incubations were similar to those seen at all longer incubation times except that the proportion of scaffold-less capsids increased to 40% or more with increasing time of incubation. The rate at which scaffold-less capsids appeared was found to be increased with increasing reaction temperature. When capsids were formed at 37°C rather than 28°C, for example, nearly all capsids were scaffold-less and polyhedral after eight hours of incubation (Figure 1e).

Maturation of closed spherical capsids into polyhedral capsids

The presence of a high proportion of closed spherical capsids in 90 minute incubations made it possible to test directly for their ability to mature into the polyhedral form. Closed spherical capsids were first precipitated from 90 minute reaction mixtures, which contain closed spherical capsids as virtually the only capsid assembly product present (see Figure 1c). Precipitates were then re-suspended in PBS containing 20 mM $MgCl_2$, incubated at 37°C, and examined (by electron microscopy) as a function of incubation time for the presence of polyhedral capsids as described in Materials and Methods.

Images of thin sectioned preparations showed that polyhedral capsids appeared promptly after the beginning of incubation at 37°C, the first ones being seen after 30 minutes. By four hours, nearly

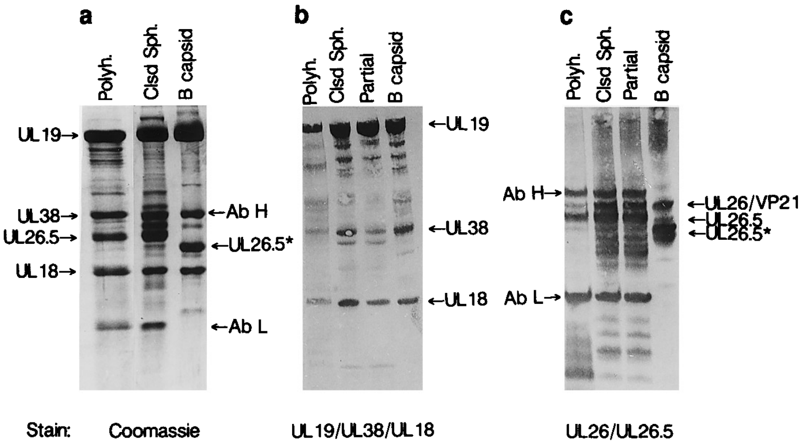

Figure 5. Protein composition of partial capsids, closed spherical capsids and polyhedral capsids. Assembly products were isolated from cell-free reaction mixtures incubated at 28°C for one minute (partial capsids), 90 minutes (closed spherical capsids), and eight hours (polyhedral capsids). The Materials and Methods section describes the procedures employed for SDS-polyacrylamide gel electrophoresis followed by Coomassie staining (a), and for SDS-polyacrylamide gel electrophoresis followed by immunoblotting (b and c). Two immunoblots were probed, respectively, with rabbit polyclonal antibodies specific for UL19, UL38 and UL18 (b), and a mouse monoclonal antibody specific for UL26 / UL26.5 (c). As a control, HSV-1 B capsids (rightmost column in each set) were analyzed together with the products of cell-free assembly. UL26.5* indicates the position of the mature (cleaved) form of the UL26.5 gene product. Note that most of the scaffolding protein (UL26.5) is present in the uncleaved form in all products of cell-free assembly. The Coomassie-stained gel of partial capsids was similar to that shown in a for closed spherical capsids. Ab H and Ab L indicate the positions of antibody heavy and light chains, respectively.

all capsids were polyhedral with scaffold material absent (Figure 4b). In contrast, only closed spherical capsids were observed in precipitates not incubated at 37°C (Figure 4a). Examination of negatively stained preparations of four hour capsids confirmed their polyhedral morphology (Figure 2d; compare Figure 2b). The presence of $MgCl_2$ was not required for the conversion of closed spherical capsids to polyhedral capsids. Similar transformation was observed when 50 mM EDTA was substituted for $MgCl_2$, but a longer incubation time was required (data not shown).

Protein composition of assembly products

The protein composition of assembly products was examined by SDS-polyacrylamide gel electrophoresis followed by Coomassie staining or immunoblotting as described in Materials and Methods. The results (Figure 5) showed that all three products of cell-free assembly contain the proteins coded by genes UL19, UL38, UL18 and UL26.5. The relative amounts of UL19, UL38 and UL18 in the cell-free assembly products were generally similar to the proportions present in B capsids isolated from HSV-1-infected cells (compare the B capsid lane in Figure 5a or b with the other lanes). VP21 was also observed in each structure (Figure 5c), the amount being greater in partial capsids and closed spherical capsids than in polyhedral capsids. In all assembly products, the scaffolding protein (UL26.5 gene product) was found to be predominantly in its immature, uncleaved form. The amount of scaffolding protein present was greater in partial capsids and closed spherical capsids than in polyhedral capsids (compare the relevant lanes in Figure 5a and c). Among polyhedral capsids, the amount of scaffolding protein present was found to decrease with increasing incubation time at 28°C the amount being greater, for example, after eight hours of incubation than after 12 hours (data not shown).

Capsid stability at low temperature

In handling capsid assembly products, we observed that whereas polyhedral capsids could be maintained at refrigerator or ice bucket temperature (2°C), closed spherical capsids were unstable. To illustrate this instability, a cell-free capsid assembly reaction was constituted and incubated under conditions (90 minutes at 28°C) in which the primary assembly product was closed spherical capsids. After precipitation with monoclonal antibody 6F10, capsids were re-suspended in 100 μl PBS and divided into two samples which were

Table 2. Effect of incubation at 2°C on the integrity of capsids assembled in the cell-free system

	Capsids per electron microscope field	
Incubation for two hours at	Closed spherical capsids	Polyhedral capsids
22°C	105 ± 13 (n = 4)	120 ± 30 (n = 3)
2°C	0 (n = 4)	131 ± 16 (n = 4)

Two cell-free capsid assembly reaction mixtures were constituted and incubated under conditions expected to yield primarily closed spherical capsids (90 minutes at 28°C) and polyhedral capsids (five hours at 37°C), respectively. Assembly products were then precipitated from each with monoclonal antibody 6F10 as described in Materials and Methods. The precipitates were re-suspended in 100 μl PBS and divided into two samples, which were incubated for two hours at 22°C (room temperature) and 2°C, respectively. After incubation, a sample of each reaction mixture was applied to an electron microscope grid, negatively stained with 1% uranyl acetate and examined in the electron microscope as described in Materials and Methods. Counts of the number of capsids per microscope field were performed with electron microscope negatives, which were recorded at a magnification of 10,000×.

incubated for two hours at 22°C (room temperature) and 2°C (slushy ice), respectively. Incubated samples were then negatively stained with uranyl acetate and examined in the electron microscope where the number of capsids per microscope field was recorded. The results (Table 2) showed that whereas closed spherical capsids were observed in the sample maintained at 22°C, no capsids at all were seen in the 2°C sample. Material in the electron microscope fields was not recognizable as capsids or as any obvious disassembly product of capsids. In contrast, when similar experiments were carried out with polyhedral capsids, incubation at 2°C did not affect capsid integrity (Table 2). Polyhedral capsids had the same morphology before as after incubation at 2°C, a behavior also characteristic of HSV-1 B capsids (data not shown).

Discussion

Experimental design

The cell-free capsid assembly experiments described here were carried out using the gene products previously shown to be required for HSV-1 capsid assembly and the same reaction conditions described previously (Newcomb *et al*., 1994). VP26 was not included in the capsid assembly reactions examined here because it is not required for formation of closed capsids (Thomsen *et al*., 1994; Tatman *et al*., 1994) and because it is not incorporated into capsids assembled in the cell-free system (Newcomb *et al*., 1994). The main difference from the previous study was the routine use of antibody precipitation rather than sucrose density gradient ultracentrifugation for the isolation of assembly products. Use of antibody precipitation in this way may have enhanced the yield of fragile assembly intermediates such as closed spherical capsids, which are not stable during sucrose gradient centrifugation or other isolation procedures carried out at 2°C (see Table 2). Antibody precipitation, however, was not required for the identification of assembly intermediates. Closed spherical capsids and partial capsids were observed when assembly products were harvested by ultracentrifugation and examined by electron microscopy of thin-sectioned specimens (W.W.N. & F.L.H., unpublished observation).

Partial capsids

The studies described here resulted in identification of two intermediates, the partial capsid and the closed spherical capsid, in HSV-1 capsid assembly. Partial capsids were the first structures recognizable in reaction mixtures as assembly products. They were arc-shaped structures consisting of a region of capsid shell, assumed to be composed of VP5, VP19C and VP23, partially surrounding a region of scaffold, most probably composed of the scaffolding protein and VP21. Partial HSV-1 capsids are suggested to be intermediates in assembly because, after their formation during the first minute of incubation, they were progressively lost from reaction mixtures and their loss correlated with an increase in the proportion of closed spherical capsids. Partial capsids did not accumulate with increasing incubation time as would be expected if they were developmental dead ends or if they resulted from degradation of more mature structures. Two-shelled wedges or angular segments comparable in morphology to HSV-1 partial capsids are observed as intermediates during *in vitro* prohead assembly in *Salmonella typhimurium* phage P22 (Prevelige *et al*., 1993).

Two aspects of partial capsid structure provide clues about how capsid assembly may take place. First, regardless of the degree of completeness of individual partial capsids (i.e. whether they were only a small wedge or a nearly complete capsid) the amount of shell present was nearly always proportional to the amount of scaffold. This observation is most consistent with the view that growth of the nascent capsid occurs by progressive addition of small, proportional amounts of scaffold and shell material rather than by decoration of a preformed scaffold with shell proteins. The suggested mechanism is similar to that thought to occur in phage P22 (Prevelige & King, 1993; Prevelige *et al*., 1993) where capsid assembly is considered to involve the addition of small oligomers containing both the shell (gp5) and scaffolding (gp8) proteins to the edges of the growing capsid. Assembly of the phage T4 procapsid is considered to follow a similar course, although progressive decoration of the complete scaffold with the major capsid protein may also occur (van Driel & Couture, 1978; Kuhn *et al*., 1987; Black *et al*., 1994).

Second, in some images of negatively stained partial capsids it could be seen that the shell layer contained capsomers (Figure 2a, arrow). Capsomers were not resolved in all images of partial capsids and it could not be determined whether the capsomers seen were hexons or pentons. The presence of capsomers in partial capsids, however, indicates that capsomers can become organized in the shell layer before it is closed and suggests that this may be the usual course of events during capsid assembly.

It is of interest to note that the triplex proteins UL38 and UL18 are present in partial capsids (Figure 5). Their presence is consistent with the earlier demonstration that capsid assembly requires both proteins (Newcomb *et al*., 1994; Tatman *et al*., 1994; Thomsen *et al*., 1994). Further, it suggests that triplexes or structures that mature into them are present in partial capsids. The presence of VP19C and VP23 in partial capsids demonstrates that the two proteins are added to nascent capsids concomitantly with VP5 and the scaffolding protein, and not, for example, at a later time after addition of VP5 and the scaffolding protein is complete. Capsid assembly in the cell-free system, therefore, appears to proceed by an incremental and progressive addition of all four major proteins (VP5, VP19C, VP23 and the scaffolding protein) examined here. We never observed assembly products that lacked any of the four proteins.

The capsid shells of some dsDNA bacteriophages contain accessory or decoration proteins (such as the T4 phage soc and hoc proteins and the gpD protein of phage λ; Casjens & Hendrix, 1988) that resemble the HSV-1 triplex proteins in that they are located on the outer surface of the capsid icosahedron at local 3-fold positions among groups of three capsomers (Yanagida, 1977; Aebi *et al*., 1977; Imber *et al*., 1980). Decoration proteins function to stabilize the capsid shell (Sternberg & Weisberg, 1977; Ishii *et al*., 1978; Steven *et al*., 1992), and it is reasonable to suggest that the HSV-1 triplex proteins may serve the same purpose. HSV-1 triplex proteins differ from phage decoration proteins, however, in that whereas VP19C and VP23 are required for capsid formation, phage decoration proteins are not essential. Further, VP19C and VP23 are added as the HSV-1 capsid shell is formed (as reported here) while phage decoration proteins are added after the basic shell architecture is complete (Ishii & Yanagida, 1975; Casjens & Hendrix, 1988).

Closed spherical capsids

After partial capsids, closed spherical capsids were the next recognizable intermediate to appear in assembly reaction mixtures. In electron micrographs, closed spherical capsids resembled mature HSV-1 capsids in overall size and in the morphology of the scaffold and shell layers (compare Figure 1c and d). They differed in that they were found to be spherical rather than icosahedral in shape and they were disassembled during incubation at 2°C while mature HSV-1 capsids were not. The properties of closed spherical capsids suggest they may play the same role in capsid formation as the proheads (also called procapsids or preheads) observed during the maturation of dsDNA bacteriophages such as λ, T4 and P22 (Hendrix, 1985; Black, 1988; Casjens & Hendrix, 1988). Proheads are spherical intermediates in phage capsid assembly. They lack DNA, but contain scaffolding protein (in cases where the phage has a scaffolding protein) and mature into polyhedral phage heads. Conversion of the prohead to the mature head is generally accompanied by a significant increase in capsid diameter, a process called prohead expansion (Casjens & Hendrix, 1988). Although there is variability in the structural stability of proheads from different phages, in some cases proheads are known to be more sensitive to disruption than the mature capsid shell. Proheads of phage T4, for instance, are more sensitive than the mature, polyhedral capsid to dissociation at 2°C or by 1% sodium dodecylsulfate (Steven *et al*., 1976). HSV-1 closed spherical capsids resemble phage proheads in most of the properties mentioned above including spherical morphology, presence of a scaffolding protein, ability to mature into polyhedral capsids and sensitivity to disruption at 2°C. Careful measurement of electron micrographs such as those shown in Figure 1c and d or 2b and c, however, showed no significant difference in diameter between HSV-1 procapsids and polyhedral capsids. Angularization of closed spherical capsids to produce polyhedral capsids, therefore, took place without a dramatic increase in shell diameter. Because of their resemblance to phage proheads, we propose HSV-1 closed spherical capsids be called ‘‘procapsids’’. We will use the term procapsids in the remainder of this discussion.

The clearest view we have of HSV-1 procapsid structure has come from the three-dimensional reconstruction (Figure 3c) computed from cryoelectron micrographs. The reconstruction confirms that the HSV-1 procapsid is spherical like bacteriophage proheads, but as described above and in contrast to the situation with bacteriophage proheads, its measured diameter (120 nm) is similar to that of the mature capsid (e.g. 125 nm measured for HSV-1 B capsids; Schrag *et al*., 1989; Booy *et al*., 1991; Zhou *et al*., 1994). Bacteriophage proheads are usually smaller in diameter than the mature capsid shell. For example, the diameters of phage P22 proheads and mature capsids are 58.0 nm and 63.0 nm, respectively (Prasad *et al*., 1993), while in phage λ (Dokland & Murialdo, 1993) they are 54.0 nm (prohead) and 63.0 nm (mature capsid).

The diameter of the HSV-1 procapsid in solution is expected to be more accurately represented by the value obtained by cryoelectron microscopy and three-dimensional image reconstruction (120 nm) than in thin sectioned (103.6 ± 4.0 nm) or negatively stained preparations because specimens preserved in the frozen-hydrated state for cryoelectron microscopy do not experience the drying-induced shrinkage involved in the other methods of sample preparation (Olson & Baker, 1989).

Apart from its spherical morphology, the open, porous nature of the procapsid is perhaps its most apparent structural difference from the mature HSV-1 capsid. The axial channels of procapsid capsomers lack the constrictions found in the mature capsid (Conway *et al*., 1994; Zhou *et al*., 1994), and there are additional holes between capsomers. The openness of the HSV-1 procapsid structure distinguishes it from bacteriophage proheads whose shells appear to have at most relatively small holes (Dokland & Murialdo, 1993; Prasad *et al*., 1993; Conway *et al*., 1995; Marvik *et al*., 1995).

Material at the triplex positions is resolved in the three-dimensional reconstruction of the procapsid (Figure 3c). We assume this material consists of VP19C and VP23 organized into triplexes or structures that mature into them. The candidate triplexes make contact with the capsomers as would be expected if they were involved in stabilization of the procapsid structure, a role that

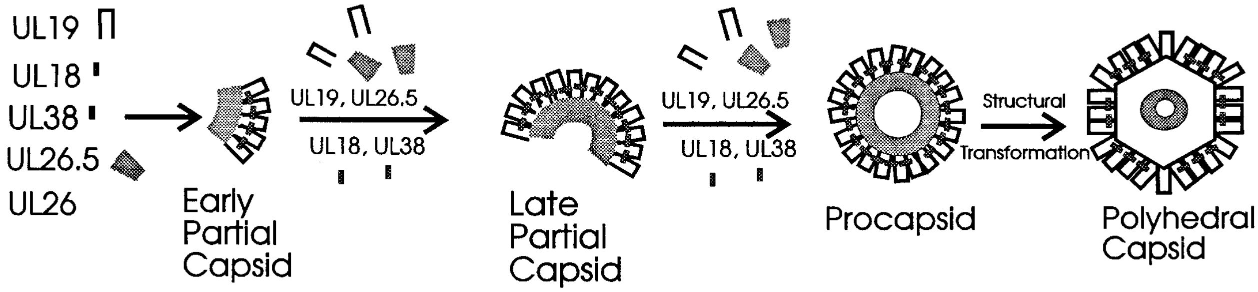

Figure 6. Schematic drawing of the pathway proposed for *in vitro* assembly of the HSV-1 capsid. Assembly begins with an early partial capsid, which enlarges to form a late partial capsid, finally closing to create the spherical closed capsid or procapsid. The procapsid then angularizes to form the mature polyhedral capsid. To conform to the results shown in Figure 5, all capsid intermediates are suggested to contain all the proteins (products of genes UL19, UL18, UL38, UL26.5 and UL26) involved in capsid assembly. Note that the transformation of procapsids to polyhedral capsids does not involve attachment of additional capsid protein molecules. Although the maturational protease (VP24) is involved in capsid assembly *in vivo*, no role for it is shown here because (as described in the Discussion) it was not present in capsids assembled *in vitro*.

would account for the observation that VP19C and VP23 are required for HSV-1 capsid formation (Thomsen *et al*., 1994; Tatman *et al*., 1994; Newcomb *et al*., 1994).

In dsDNA bacteriophage, the prohead is the receptacle in which DNA packaging is initiated. In most cases the prohead converts to the mature polyhedral form after initiation of DNA packaging (Bjornsti *et al*., 1983; Hohn, 1983; Black, 1988). In view of the other similarities between HSV-1 procapsids and phage proheads, it is attractive to speculate that procapsids may be involved in the initial stages of HSV-1 DNA packaging.

Polyhedral capsids

Polyhedral (icosahedral) capsids predominated in reaction mixtures incubated for five hours or longer. The exact time of their appearance varied somewhat from one experiment to another, the shortest times being three to four hours and the longest about ten hours. Formation of polyhedral capsids was more rapid and predictable when incubations were carried out at 37°C rather than 28°C, and 37°C incubations yielded a higher proportion of polyhedral capsids lacking scaffolds (as judged by electron microscopy). Capsids containing scaffolds always appeared in reaction mixtures before the scaffold-less form, suggesting that capsids are assembled with a scaffold, which is later lost.

Experiments to test the ability of purified procapsids to mature into the polyhedral form (Figure 4) were carried out to address the formal possibility that procapsids and polyhedral capsids may have arisen by independent pathways rather than by a precursor-product relationship. The results showing that a homogeneous population of polyhedral capsids was obtained from purified procapsids demonstrated that procapsids can be transformed into polyhedral ones. Transformation took place under conditions in which no free capsid proteins were available for new capsid formation. The results are therefore most consistent with a precursor-product pathway, and they suggest that the transformation involves only the proteins originally present in the procapsid.

Figure 6 illustrates the pathway of capsid formation suggested by the present results. Mature polyhedral capsids are shown to be formed in a pathway involving partial capsids and procapsids as intermediates. In agreement with information about the protein composition of assembly intermediates (Figure 5), capsids at all stages of assembly are shown to contain all the proteins involved in capsid assembly. Morphological transformation of the procapsid to the polyhedral capsid is shown to take place without addition of further protein subunits as suggested by the results of the experiment shown in Figure 4.

Capsid protein composition

The protein composition of capsids and capsid assembly intermediates was determined by SDS-polyacrylamide gel electrophoresis followed by Coomassie staining and western immunoblotting beginning with specimens harvested by precipitation with monoclonal antibody 6F10. The specimens analyzed therefore all contained antibody heavy and light chains (indicated in Figure 5). In principle, the composition of assembly products determined in this way could be affected by the co-precipitation of VP5-containing complexes not identifiable as assembly intermediates. We note, however, that the protein composition of polyhedral capsids as reported here (Figure 5a) is in qualitative agreement with the composition of similar capsids prepared in an earlier study without the use of antibody precipitation (Newcomb *et al*., 1994). The absence of significant amounts of non-HSV-1 (or antibody) proteins from the analyses shown in Figure 5a suggests that Sf9 cell proteins were largely excluded from antibody precipitates of capsid assembly intermediates.

Scaffolding protein

The scaffolding protein was found to be present in all three capsid assembly intermediates (i.e. partial capsids, procapsids and polyhedral capsids) examined in this study. Despite the presence in reaction mixtures of the enzyme (the maturational protease) that normally cleaves it, however, all or nearly all of the scaffolding protein was found to be in the immature, uncleaved form (Figure 5a). We suggest the above observation may be related to the fact that in the Sf9 cell extracts employed here the UL26 gene product has been cleaved at the R site separating VP24 and VP21 (W.W.N. & F.L.H., unpublished observation). Without its VP21 moiety, whose 25 amino acid C terminus can bind VP21 to the VP5-based capsid shell (Kennard *et al*., 1995; Matusick-Kumar *et al*., 1995b; Thomsen *et al*., 1995; Hong *et al*., 1996), the proteolytically active portion of the UL26 gene product (VP24) may lack a way to become associated with nascent capsids and therefore be unable to cleave capsid-associated scaffolding protein. In agreement with the above suggestion, VP24 was not detected when procapsids were tested for its presence by western immunoblotting using a VP24-specific antibody (W.W.N. & F.L.H., unpublished observation). The presence of uncleaved scaffolding protein in polyhedral capsids indicates that conversion of procapsids to polyhedral capsids does not require cleavage of the scaffolding protein. Formation of polyhedral capsids containing uncleaved scaffolding protein is consistent with earlier observations demonstrating that stable, icosahedral capsids can form in the absence of the maturational protease (Thomsen *et al*., 1994; Newcomb *et al*., 1994).

The present results are the first to demonstrate that stable, polyhedral capsids lacking scaffold material can be formed in the absence of the maturational protease and without cleavage of the scaffolding protein (Figure 1d, e, 4b and 5a). To account for this observation, we suggest that maturation of the procapsid to the polyhedral form is accompanied by a loss of the VP5 site (e.g. by a change in protein conformation) that binds scaffolding protein in the procapsid. Loss of the VP5 site would permit exit of uncleaved scaffolding protein from the capsid cavity, which *in vitro* is observed to take place slowly (four to 18 hours) at 28°C and more rapidly at 37°C (Figure 4). According to the above suggestion, the required role for the protease in capsid assembly (Preston *et al*., 1983) would be to cleave the scaffolding protein at the R site as recently suggested by Robertson *et al*. (1996) or perhaps to cleave a different capsid protein.

Relationship of cell-free to *in vivo* capsid assembly

Studies of HSV-1 capsid assembly in the cell-free system were undertaken with the idea that they would clarify assembly as it occurs in infected cells. As with all *in vitro* studies, the results need to be interpreted with caution and, insofar as is possible, with reference to similar *in vivo* experiments. Previous analyses of HSV-1 capsids assembled *in vitro* demonstrated that morphologically they are a faithful representation of the B capsids found in the nuclei of HSV-1-infected cells (Newcomb *et al*., 1994). For example, *in vitro* assembled capsids were found to be a uniform population of $T = 16$ icosahedra with the same diameter as B capsids and a similar protein composition. Capsids assembled *in vitro* migrated at the same rate as B capsids during sucrose density gradient ultracentrifugation. A significant difference between cell-free-assembled and *in vivo* capsids is the presence of the dispensable minor capsid protein VP26 in the latter but not the former (Newcomb *et al*., 1994). Preliminary experiments in our laboratory have suggested the absence of VP26 from *in vitro* capsids may be due to binding of VP26 to sedimentable material in cell-free extracts (W.W.N. & F.L.H., unpublished observations). VP26 may therefore not be available for incorporation into capsids in the *in vitro* system.

In its basic features, capsid formation in the cell-free system resembles assembly as it occurs in insect cells multiply infected with recombinant baculoviruses (Thomsen *et al*., 1994; Tatman *et al*., 1994). For instance, assembly in both systems requires VP5, VP19C, VP23 and either the scaffolding protein or the maturational protease. VP26 is not required for capsid formation in either system. The morphology of capsids formed in the two systems is indistinguishable in electron micrographs and the two are compositionally similar (except for the issue involving VP26 noted above). Perhaps the most noteworthy difference is the fact that the scaffold is absent in capsids formed during incubation at 37°C or during prolonged incubation at 28°C *in vitro*. Scaffold-less capsids have not been reported in the recombinant baculovirus/insect cell system (Thomsen *et al*., 1994, 1995; Tatman *et al*., 1994).

The identification of partial capsids and procapsids during assembly of HSV-1 capsids *in vitro* suggests that similar structures may be found in HSV-1-infected cells. In fact, partial capsidlike structures have been reported by Nii *et al*. (1968). Using electron micrographs of HSV-1-infected FL cells, these authors observed two-shelled, arc-shaped structures morphologically resembling partial capsids in the cell nuclei. These structures were observed only at early times after the initiation of infection, but no effort was made to demonstrate that they could mature into closed capsids. In contrast to the situation with partial capsids, there have as yet been no reports of structures with the properties of procapsids in HSV-1-infected cells. There are two reasons to believe procapsids may be present but not yet detected. (1) It is very difficult to distinguish procapsids from B capsids in electron micrographs of thin-sectioned preparations. Both contain scaffold and shell layers that are similar in morphology and the distinction between spherical and icosahedral shape is difficult to judge in thin-sectioned preparations. Procapsids may therefore be present, but mistaken for B capsids, which abound in the nuclei of infected cells. (2) The fragility of procapsids during incubation at 2°C may have affected attempts to isolate them biochemically as most cell fractionations are performed at or near 2°C. It may therefore be productive to make further attempts to identify procapsids in the nuclei of HSV-1-infected cells.

The properties of procapsids suggest that this may be the capsid type that accumulates when certain mutant HSV-1 strains are grown under non-permissive conditions. Capsids that accumulate in cells infected with *ts*1201 (Preston *et al*., 1983) or *m*100 (Gao *et al*., 1994), for example, yield images in electron micrographs that are indistinguishable from those reported here for procapsids. The difficulties reported (Matusick-Kumar *et al*., 1995a) in the biochemical isolation of such capsids is consistent with the view that they may be procapsids that are disassembled at 2°C during standard capsid isolation procedures.

Materials and Methods

Cell-free capsid assembly

Thomsen *et al*. (1994) described construction of the five recombinant baculoviruses (containing HSV-1 genes UL18, UL19, UL26, UL26.5 and UL38, respectively) employed in this study. Suspension cultures of *Spodoptera frugiperda* (Sf9) cells were infected (multiplicity of infection = 5) with individual recombinant baculoviruses as described previously (Summers & Smith, 1987; Thomsen *et al*., 1994), and infection was continued for a total of 64 hours at 28°C. Thereafter cells were harvested by centrifugation, frozen, diluted 1:3 (based on pellet volume) with PBS and frozen in 1 ml aliquots.

Cell free capsid assembly was carried out by a modification of the procedure described previously (Newcomb *et al*., 1994). Assembly-competent reaction mixtures were constituted by mixing aliquots of the five infected cell suspensions described above at 4°C; typical reactions contained 75 μl of cells expressing UL19 and 25 μl of cells expressing each of the four remaining genes (i.e. UL18, UL26, UL26.5 and UL38). Cells were then lysed by three cycles of freezing and thawing, clarified by centrifugation at 16,000 ***g*** for one minute and the supernatant adjusted to 50 mM EDTA, 5 mM DTT. The total protein concentration was 15 to 20 mg/ml at this stage. Extracts were incubated at 28°C for one minute to 18 hours as indicated in the text, centrifuged for 30 seconds at 16,000 ***g*** and treated with 0.1 volume of monoclonal antibody 6F10 (4 mg/ml) to precipitate capsids and capsid-related structures. Precipitates were allowed to form during a 15 minute incubation at room temperature, harvested by centrifugation for two minutes at 16,000 ***g*** and processed for electron microscopy or SDS-polyacrylamide gel electrophoresis as described below. Reaction mixtures incubated for longer than five hours were made 1% in Triton X-100 before precipitation with monoclonal antibody 6F10. This treatment prevented capsid assembly products from being included in non-specific precipitates of Sf9 cell proteins that formed after prolonged (five hours or longer) incubation of reaction mixtures at 28°C. 1% Triton X-100 did not affect the morphology of the assembly intermediates (i.e. partial capsids and procapsids) described in this study. The efficiency of capsid formation was estimated by the proportion of added VP5 incorporated into capsids which was approximately 60% in reactions incubated for two hours or longer (Newcomb *et al*., 1994). Capsid assembly depended on incubation of reaction mixtures at elevated temperature (i.e. 28°C or higher). No capsids or other precipitable assembly products formed if extracts were maintained at 4°C.

Monoclonal antibody 6F10

A mouse hybridoma cell line secreting monoclonal antibody 6F10 was isolated by the method of Chapman *et al*. (1984). BALB/c mice were immunized with purified HSV-1 B capsids (Newcomb & Brown, 1991); spleen cells from immune mice were fused with Sp2/O-Ag14 myeloma cells (Shulman *et al*., 1978); and cloned hybridoma cell lines secreting specific monoclonal antibodies were identified by ELISA assay (Engvall & Perlman, 1971) using B capsids as antigen. Experiments were performed with purified 6F10 antibody, which was prepared by growing the cloned hybridoma cell line as an ascites tumor in BALB/c mice. The antibody was purified from ascites fluid by adsorption and elution from a Protein G-Sepharose column (Pharmacia), and stored frozen (–20°C) at a concentration of 4 mg/ml. Antibody 6F10 (IgG2b subclass) was found to precipitate HSV-1 B capsids and to recognize VP5 specifically in an immunoblot (Bloodgood *et al*., 1986) performed with B capsid proteins.

Electron microscopy

Precipitates containing capsid assembly products were prepared for electron microscopy by fixation, embedding in Epon 812 and sectioning as previously described (Matusick-Kumar *et al*., 1994) except that fixation was carried out in 5% (w/v) glutaraldehyde for 24 hours at room temperature. Negative staining was performed with 1% (w/v) uranyl acetate (Thomas *et al*., 1985). All thin section and negative stain electron micrographs were recorded on a JEOL 100CX transmission electron microscope operated at 80 keV. Measurements of capsids and capsid-related structures in electron micrographs were made with positive prints in which the total magnification was 100,000× or greater. Methods employed for cryoelectron microscopy and three-dimensional image reconstruction are described in the accompanying paper (Trus *et al*., 1996).

SDS-polyacrylamide gel electrophoresis and western immunoblotting

Previously described procedures were employed for SDS-polyacrylamide gel electrophoresis followed by Coomassie staining (Newcomb *et al*., 1993) and for SDS-polyacrylamide gel electrophoresis followed by western immunoblotting (Thomsen *et al*., 1995). Immunoblots were stained with: (1) polyclonal rabbit antisera specific for UL19, UL38 and UL18 (generously donated by Drs G. Cohen and R. Eisenberg); or (2) a mouse monoclonal antibody (MCA406; Serotec, Washington, DC) specific for UL26 and UL26.5. Rabbit antisera were used at a dilution of 1:10,000 and detection of immune complexes was performed with an alkaline phosphatase color reaction (Thomsen *et al*., 1995). HSV-1 B capsids, used as a standard in immunoblotting studies, were prepared as previously described (Newcomb *et al*., 1993) from BHK-21 cells infected with the 17MP strain of HSV-1.

Acknowledgements

We thank Gary Cohen and Roselyn Eisenberg (University of Pennsylvania) for providing rabbit antisera, and Stéphanie Gaillard for technical assistance. This work was supported by awards from the National Science Foundation (MCB-9417770) and the National Institutes of Health (AI-137549).

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Edited by M. Gottesman

(Received 12 March 1996; received in revised form 30 July 1996; accepted 19 August 1996)